RNA interference targeting Fas protects mice from fulminant hepatitis

ERWEI SONG[1,2], SANG-KYUNG LEE[1], JIE WANG[2], NEDIM INCE[1], NENGTAI OUYANG[3], JUN MIN[2], JISHENG CHEN[2], PREMLATA SHANKAR[1] & JUDY LIEBERMAN[1]

[1]*Center for Blood Research and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA*
[2]*Department of Hepatobiliary Surgery, Sun-Yat-Sen Memorial Hospital, Guangzhou, China*
[3]*Department of Cell Pathology, Guangzhou Medical Institute, Guangzhou, China*
E.S. and S.-K.L. contributed equally to this study.
Correspondence should be addressed to J.L.; e-mail: lieberman@cbr.med.harvard.edu

Published online: 10 February 2003; corrected 18 February 2003 (details online); doi:10.1038/nm828

© 2003 Nature Publishing Group http://www.nature.com/naturemedicine

RNA interference (RNAi) is a powerful tool to silence gene expression post-transcriptionally[1]. However, its potential to treat or prevent disease remains unproven. Fas-mediated apoptosis is implicated in a broad spectrum of liver diseases, where inhibiting hepatocyte death is life-saving[2]. We investigated the *in vivo* silencing effect of small interfering RNA (siRNA) duplexes targeting the gene *Fas* (also known as *Tnfrsf6*), encoding the Fas receptor, to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis. Intravenous injection of *Fas* siRNA specifically reduced *Fas* mRNA levels and expression of Fas protein in mouse hepatocytes, and the effects persisted without diminution for 10 days. Hepatocytes isolated from mice treated with *Fas* siRNA were resistant to apoptosis when exposed to Fas-specific antibody or co-cultured with concanavalin A (ConA)-stimulated hepatic mononuclear cells. Treatment with *Fas* siRNA 2 days before ConA challenge abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases. Administering *Fas* siRNA beginning one week after initiating weekly ConA injections protected mice from liver fibrosis. In a more fulminant hepatitis induced by injecting agonistic Fas-specific antibody, 82% of mice treated with siRNA that effectively silenced *Fas* survived for 10 days of observation, whereas all control mice died within 3 days. Silencing Fas expression with RNAi holds therapeutic promise to prevent liver injury by protecting hepatocytes from cytotoxicity.

RNAi by synthetic siRNAs 21–23 nucleotides in length silences cellular and viral gene expression in mammalian cells *in vitro*[3,4]. siRNA duplexes pulse-injected into the tail vein of mice inhibit expression of a co-transfected firefly luciferase gene[5,6]. Viral-mediated delivery of siRNA *in vivo* reduces exogenous green fluorescent protein (GFP) and endogenous glucuronidase expression[7]. In these studies, RNA silencing was prominent in the liver, indicating that the liver is an ideal organ to test the therapeutic potential of siRNA. Hepatocytes are very susceptible to Fas-mediated apoptosis because they highly express Fas[8]. As a consequence, Fas-mediated apoptosis is important in hepatic injury from diverse insults, including viruses, autoimmunity and transplant rejection[2,9]. Fas-deficient *lpr* mice survive challenge with factors that induce fulminant hepatitis in normal mice [10,11] and show reduced fibrosis after chronic hepatic insults[12]. Therefore, we investigated whether intravenous siRNA injection targeting *Fas* could inhibit Fas expression on mouse hepatocytes *in vivo* and protect the liver from fulminant hepatitis and fibrosis.

First, we verified delivery of synthetic siRNA duplexes into mouse hepatocytes *in vivo* by hydrodynamic tail vein injection of Cy5-labeled *Fas*(sequence 1) siRNA (50 μg, 2.0–2.5 mg/kg)[13] (for details of *Fas* target sequences, see Methods). Twenty-four hours after the last of three injections, 88 ± 6% of hepatocytes had taken up the siRNA and were Cy5-positive as determined by flow cytometry (Fig. 1*a*). The efficient delivery confirms that siRNA duplexes can be taken up by most liver cells *in vivo*[5,6]. Transduction efficiency was higher than the 40% efficiency observed with a single injection of reporter plasmid DNA[13]. Measuring uptake of Cy5–*Fas* siRNA is only a surrogate for measuring *Fas* siRNA uptake *in vivo*, however, because siRNA labeled with Cy5 at the 3′ end does not induce silencing (data not shown). *Fas* mRNA and protein expression in hepatocytes was measured by RNase protection assay (RPA) and immunoblotting, respectively, at various times after injection. Treatment with *Fas*(sequence 1) siRNA reduced *Fas* mRNA expression eight to tenfold as compared to saline or *GFP* siRNA injection (ratios of *Fas* signal to *Gapdh* signal (internal control): 0.0024 ± 0.0004 versus 0.022 ± 0.002 saline or 0.022 ± 0.002 *GFP* (sequence 1) siRNA, $n = 3$ per group, $P < 0.001$ compared with either control), as measured 24 h after the last injection (Fig. 1*b*). Immunoblot analysis showed that *Fas*(sequence 1) siRNA reduced Fas protein in hepatocytes nearly to background (Fig. 1*c*). The effect was specific, as injection of control siRNA targeting *GFP* did not change Fas expression, and *Fas*(sequence 1) siRNA treatment did not affect the expression of other *Fas*-related genes, such as those encoding FasL, FADD, FAF, TRAIL and TNF receptor p55 and RIP (Fig. 1*b*). Specificity was also demonstrated by RPAs of hepatocytes from mice injected with siRNAs targeting other regions of *Fas*. Two additional siRNAs, sequences 5 and 6, silenced Fas expression by ~81–86%, as efficiently as sequence 1. In contrast, sequence 2, beginning only one nucleotide downstream of sequence 6, reduced Fas expression by only 38% (Fig. 3*e*). Two sequences (3 and 4) did not suppress Fas expression at all.

siRNA seemingly suppresses expression more efficiently than does the intraperitoneal injection of antisense oligodeoxynucleotide (ODN) described in a previous report[14], where mice were treated with a higher dosage (6 mg/kg) of anti-*Fas* ODN for 12 consecutive days. Approximately 14-fold less total nucleic acid was administered here (50 μg for 3 injections to mice weighing

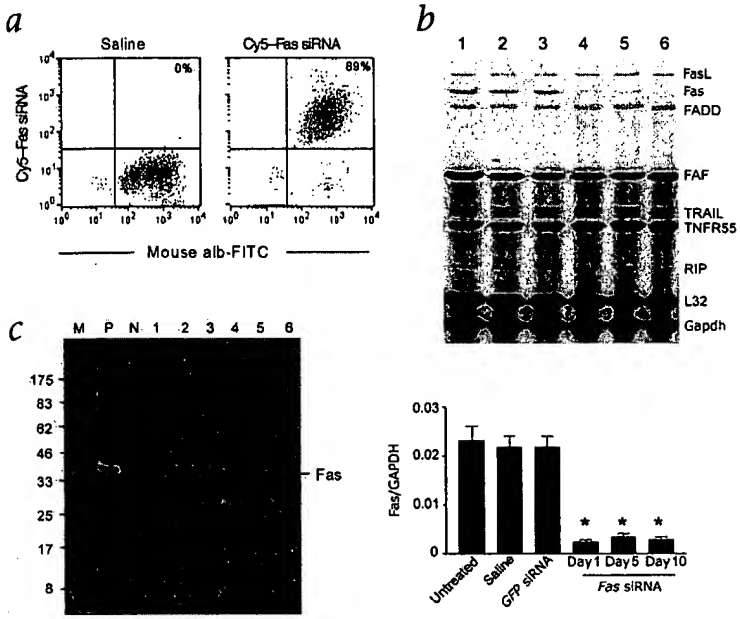

Fig. 1 Injection of siRNA duplex efficiently silences *Fas* gene expression in mouse hepatocytes. ***a***, Hepatocytes harvested 24 h after 3 injections of saline or Cy-5-labeled *Fas*(sequence 1) siRNA were stained with albumin-FITC (alb-FITC) and analyzed by flow cytometry. A high proportion of hepatocytes take up the duplex siRNA, as indicated. ***b***, RPA for *Fas* mRNA expression in hepatocytes from mice that were untreated (lane 1) or were injected 24 h earlier with saline (lane 2), *GFP*(sequence 1) siRNA (lane 3) or *Fas*(sequence 1) siRNA (lane 4). Silencing of *Fas* expression in mice treated with *Fas* siRNA is maintained 5 (lane 5) or 10 d (lane 6) later. Expression of other genes involved in the Fas pathway and housekeeping genes (*L32* and *Gapd*) were unaffected (names of the corresponding proteins are listed at right). Similar results were obtained in 3 independent experiments. The graph shows results of densitometric quantification of the *Fas*/*Gapdh* ratios in 3 mice per condition. *Fas* mRNA levels in hepatocytes are significantly lower (*, $P < 0.001$) at all times in mice treated with *Fas* siRNA mice than in control mice. ***c***, Fas immunoblot of lysates from hepatocytes obtained from untreated mice (lane 1), or 24 h after saline (lane 2), *GFP* siRNA (lane 3) or *Fas* siRNA (lane 4) injection, and 5 (lane 5) or 10 d (lane 6) after *Fas* siRNA injection. Mouse recombinant Fas and FasL proteins serve as positive (P) and negative (N) controls, respectively. Similar results were obtained in 3 independent experiments.

© 2003 Nature Publishing Group http://www.nature.com/naturemedicine

~24 g) to reduce Fas expression to a similar degree. In a recent study, siRNA was quantitatively more efficient than antisense ODN at suppressing co-transfected GFP expression both *in vitro* and *in vivo*[15]. However, a direct comparison using varying doses is needed to determine relative efficiency.

A major concern in applying siRNA therapeutically is the stability of silencing under physiological conditions. Double-stranded siRNAs resist biodegradation in fetal calf serum and in human plasma[15], and in one study siRNA-directed suppression of a co-transfected gene *in vivo* in mouse liver was maintained for several days[6]. In our study, levels of both *Fas* mRNA and Fas protein were stably reduced for 10 days after the last injection (*Fas*/*Gapdh* mRNA ratios: day 1, 0.0024 ± 0.0004; day 5, 0.0035 ± 0.0006; day 10, 0.0029 ± 0.0004, $P > 0.05$) (Fig. 1*b* and *c*). *Fas* mRNA levels and Fas protein expression were still only 40% of those in control mice on day 14, but returned to normal 20 days after the last injection (data not shown). The duration of silencing in hepatocytes, which contrasts with more transient silencing in transformed cell lines[3], indicates that sustained therapeutic silencing in hepatocytes may not require siRNA expression from plasmids or viral vectors. The difference between liver cells and cell lines probably occurs because hepatocytes are mostly non-dividing, so there is no siRNA dilution with cell division. However, although unlikely[16], the possibility that sustained suppression in hepatocytes might be due to siRNA amplification, which occurs in lower species[17,18], has not been ruled out. Because silencing after duplex siRNA injection is prolonged but not permanent, long-term toxicity, such as lymphoproliferative or autoimmune disease, seen in humans with mutations of the homolog *FAS* (*TNFRSF6*) and in the *lpr* mouse[19] is probably of little concern.

Two known mechanisms underlie Fas-mediated fulminant hepatitis. Ligation of the Fas receptor on hepatocytes induces massive apoptosis, accompanied by an infiltration of inflammatory cells and secondary necrosis[20]. Fas engagement also provokes hepatic inflammation by inducing expression of hepatic chemokines that recruit and activate immune cells, leading to hepatocyte death in a pro-inflammatory milieu[21]. To determine whether the efficient suppression of Fas

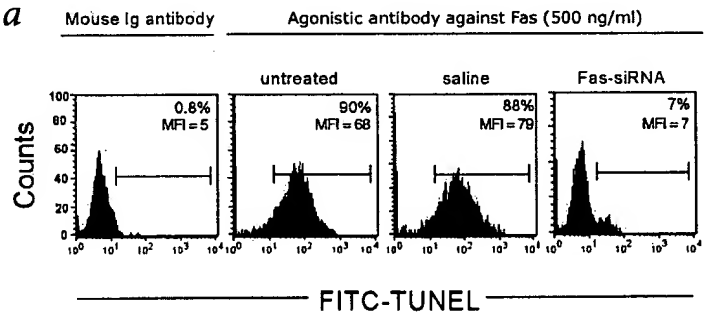

Fig. 2 *In vivo* treatment with *Fas* siRNA protects mouse hepatocytes from Fas-mediated apoptosis and cytotoxic lysis by ConA-activated hepatic mononuclear cells. ***a***, Flow cytometric analysis of FITC-TUNEL staining of primary hepatocytes from untreated mice, mice injected with saline and mice injected with *Fas*(sequence 1) siRNA, exposed *in vitro* to 500 nM of agonistic Fas-specific monoclonal antibody. Hepatocytes from untreated mice not exposed to Jo2 monoclonal antibody served as negative control. Percentage of FITC-TUNEL-positive cells and mean fluorescence intensity (MFI) are indicated. ***b***, Hepatic mononuclear cells from ConA-injected mice lyse hepatocytes from mice treated with saline (■) but not mice injected with *Fas*(sequence 1) siRNA (◆). E/T ratio, effector/target ratio. Similar results were obtained in 3 independent experiments. *, $P < 0.001$ at each ratio.

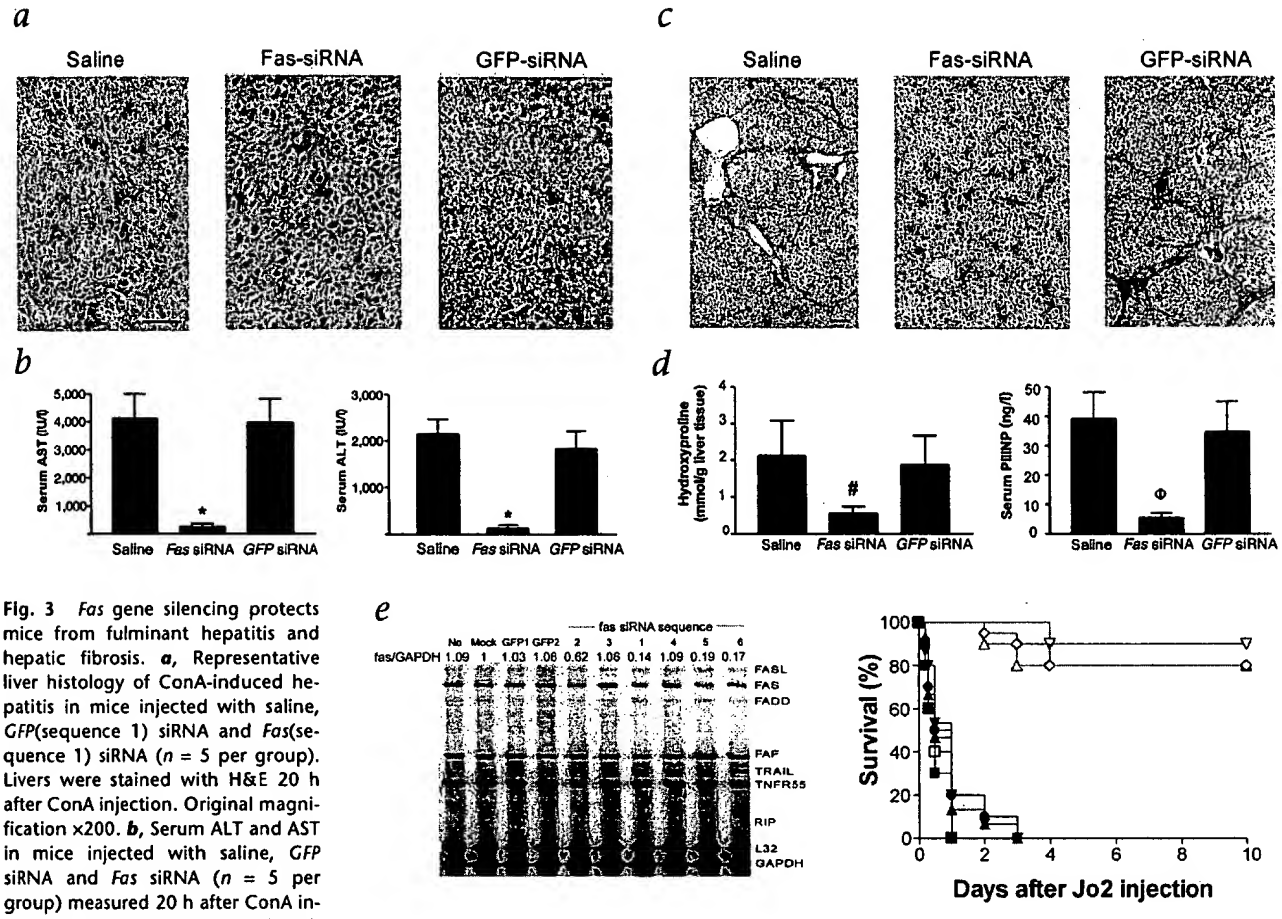

Fig. 3 *Fas* gene silencing protects mice from fulminant hepatitis and hepatic fibrosis. ***a***, Representative liver histology of ConA-induced hepatitis in mice injected with saline, *GFP*(sequence 1) siRNA and *Fas*(sequence 1) siRNA (n = 5 per group). Livers were stained with H&E 20 h after ConA injection. Original magnification x200. ***b***, Serum ALT and AST in mice injected with saline, *GFP* siRNA and *Fas* siRNA (n = 5 per group) measured 20 h after ConA injection. ***c***, Representative liver histology 1 week after 6 weekly injections of ConA in mock-treated mice and mice injected with *GFP*(sequence 1) siRNA and *Fas*(sequence 1) siRNA (n = 3 per group). Original magnification x100. Livers of mice treated with *Fas* siRNA mice did not develop bridging fibrosis. ***d***, At 1 week after the last ConA injection in the chronic hepatitis model, hepatic hydroxyproline and serum procollagen type III (PIIINP), indicators of ongoing fibrosis, were normal in mice injected with *Fas* siRNA (n = 3 per group) but elevated in mock-treated mice and those treated with *GFP* siRNA . #, $P < 0.05$; Φ, $P < 0.01$, as compared with control groups. ***e***, Survival advantage of mice injected with *Fas* siRNA as compared to saline or *GFP* siRNA after challenge by intraperitoneal injection with Fas-specific antibody and observation for 10 d before sacrifice. RPA analysis at left (representative data from 2 independent experiments) shows specific silencing of Fas expression in hepatocytes of mice treated with *Fas* siRNA sequences 1, 5, and 6 and partial silencing with Fas sequence 2. GFP1, *GFP*(sequence 1); GFP2, *GFP*(sequence 2). The *Fas*/*Gapdh* signal ratio has been normalized to that of mock-treated mice. Sequences that silenced *Fas* by ≥80% protected against fulminant hepatitis, whereas sequences that did not silence or silenced inefficiently provided no protection. *, $P < 0.0001$. ■, GFP (dT) (n = 10); ▲, GFP (CU) (n = 15); ▼, saline (n = 15); ◇, *Fas*(sequence 1) (n = 20); ●, *Fas*(sequence 2) (n = 10); □, *Fas*(sequence 4) (n = 10); △, *Fas*(sequence 5) (n = 10); ▽, *Fas*(sequence 6) (n = 10).

expression in the liver after duplex siRNA injection protects hepatocytes from Fas-mediated apoptosis, hepatocytes from mice treated with *Fas*(sequence 1) siRNA and mock-injected mice were challenged *in vitro* with an agonistic Fas-specific antibody (Jo2) or activated hepatic mononuclear cells harvested from ConA-treated mice. *In vitro* exposure of hepatocytes from untreated or mock-treated mice to Jo2 (500 ng/ml) for 24 h resulted in 87.8 ± 6.8% (n = 5) apoptotic cells as measured by fluorescein isothiocyanate–terminal deoxynucleotide transferase-mediated dUTP nick end labeling (FITC-TUNEL) staining (Fig. 2*a*). In contrast, only 7.9 ± 1.5% (n = 5) of cultured hepatocytes from mice treated with *Fas*(sequence 1) siRNA were stained by TUNEL ($P < 0.0001$). Moreover, protection from *in vitro* Fas-induced apoptosis after tail vein injection of other *Fas* siRNAs correlated with Fas suppression: *Fas* siRNA that did not silence had no effect on apoptosis, whereas partial silencing provided partial protection (data not shown). A recent study shows that FasL-expressing natural killer T cells are the hepatic mononuclear cells that induce hepatic cell injury in ConA-induced hepatitis[22,23]. We therefore tested, using an alanine aminotransferase (ALT) release assay, whether hepatocytes from mice treated with *Fas*(sequence 1) siRNA were resistant to cytolysis by hepatic mononuclear cells isolated from ConA-treated mice (Fig. 2*b*). Hepatocytes from mice treated with *Fas* siRNA were not lysed by hepatic mononuclear cells, whereas hepatocytes from mock-treated or untreated controls were. Thus, *Fas* silencing effectively inhibits hepatocyte apoptosis *in vitro*.

We next examined whether *Fas* siRNA treatment protects mice from fulminant hepatitis in two models of Fas-mediated liver damage. Mice treated with *Fas*(sequence 1) siRNA, *GFP*(sequence 1) siRNA or saline were challenged 1 day later by intravenous injection of ConA. Serum transaminase concentrations and liver

© 2003 Nature Publishing Group http://www.nature.com/naturemedicine

© 2003 Nature Publishing Group http://www.nature.com/naturemedicine

pathology were analyzed 20 h after ConA challenge. All control saline-treated mice and mice treated with *GFP* siRNA had extensive liver damage, showing confluent hepatocyte necrosis with bridging and inflammatory cell infiltrates surrounding the portal and central veins (Fig. 3*a*). Most surviving hepatocytes had cytoplasmic swelling, and there was frequent nuclear chromatin condensation, indicative of apoptosis. In contrast, pretreatment with *Fas* siRNA prevented liver cell necrosis and abrogated inflammatory infiltration, although mild hepatocyte swelling occurred. In ConA-induced hepatitis, release of the transaminases ALT and aspartate aminotransferase (AST) from damaged hepatocytes peaks in the serum 20 h after injection and is a good indicator of the extent of liver damage[24]. In agreement with the morphological findings, *Fas* siRNA treatment almost completely prevented the elevation of serum ALT (142 ± 58 IU/l versus 2150 ± 312 IU/l in saline-treated controls; $n = 5$, $P < 0.001$; normal, 30 IU/l) and AST (270 ± 90 IU/l versus 4120 ± 876 IU/l in saline-treated controls; $n = 5$, $P < 0.001$; normal, 50 IU/l) (Fig. 3*b*).

Fas-mediated hepatocyte apoptosis also contributes to the development of liver fibrosis in chronic hepatitis[12,25]. To evaluate further the therapeutic potential of *Fas* siRNA to treat chronic liver injury, and to determine whether siRNA administered after the noxious insult can protect in a more clinically relevant scenario, we delayed siRNA treatment until 24 h after the second of six weekly injections with a reduced dose of ConA. The siRNA injection was repeated once two weeks later. Mice were killed at seven weeks, one week after the last ConA injection. All mock-treated mice and mice treated with *GFP* siRNA developed bridging fibrosis in the liver parenchyma, whereas no hepatic fibrosis or necrosis was seen in mice treated with *Fas*(sequence 1) siRNA (Fig. 3*c*). *Fas* siRNA treatment also significantly reduced two chemical indicators of active fibrosis, hepatic hydroxyproline[26] (0.56 ± 0.17 mmol/g liver tissue in mice treated with *Fas* siRNA versus 2.13 ± 0.95 mmol/g in mock-treated controls; $n = 3$, $P < 0.05$; normal, 0.5 mmol/g) and serum procollagen type III (PIIINP)[27] (5.6 ± 1.5 ng/l in mice treated with *Fas* siRNA versus 39.2 ± 2.1 ng/l in saline-treated controls; $n = 3$, $P < 0.01$; normal 5 ng/l) (Fig. 3*d*). In addition, there was no evidence of toxicity, including lymphoproliferation, splenomegaly or other organ damage, from prolonged silencing of *Fas*, even with repeated injection (data not shown). These results indicate that treatment with *Fas* siRNA might provide protection even after the initiation of chronic liver injury.

To evaluate further whether *Fas* siRNA promotes survival in fulminant hepatitis, we challenged mice in a more aggressive hepatitis model[10] by intraperitoneal injection of a Fas-specific antibody. All control mice ($n = 40$) died within 3 days, mostly within 24 h after antibody injection. Mice treated with *Fas* siRNAs(sequences 2 or 4), which silenced expression by only 38% or not at all, also were not protected. However, mice pretreated with *Fas* siRNAs that silenced expression by 81–86% (sequences 1, 5 or 6) were protected from lethal challenge: 33 of 40 mice survived for the 10 days of observation (log-rank test, $P < 0.0001$) (Fig. 3*e*). Fatalities in the *Fas* siRNA–treated group were from hemorrhage secondary to liver failure. Liver damage in lethal fulminant hepatitis culminates within the first few weeks, but the survivors recover thereafter[28]. In fact, livers and other organs from the surviving mice appeared normal when the animals were killed at the end of the observation period (data not shown). Hence, *Fas* silencing during the acute insult prevents death from fulminant hepatitis.

Based on the crucial role that Fas-mediated apoptosis plays in a broad spectrum of immune-related liver diseases, siRNA-directed *Fas* silencing may be of therapeutic value for preventing and treating acute and chronic liver injury induced by viral and autoimmune hepatitis[2,9], alcoholic liver disease, acute and chronic liver failure[20,25] and rejection of liver transplants[2]. In fact, preliminary studies in a mouse transplant model indicate that prophylactic administration of siRNA might prevent rejection (J.W., N.O., J.M. and J.C., unpublished data). Protection from other hepatotoxic agents needs to be investigated. Extension of these results to prevent or treat other diseases, however, may require other strategies, such as viral vectors, to target other cell types and tissues. In addition, it is not clear whether hydrodynamic injection can be adapted to primates, as the volume injected into mice is a substantial fraction of the circulating blood volume. However, regional delivery of high concentrations of siRNA via hepatic artery or portal vein cannulation remains a viable alternative.

Methods

Preparation of siRNAs. siRNAs were synthesized using 2′-O-ACE-RNA phosphoramidites (Dharmacon Research, Lafayette, Colorado). The sense and anti-sense strands of siRNAs were: *Fas*(sequence 1), beginning at nt 364, 5′- P.GUGCAAGUGCAAACCAGACdTdT-3′ (sense), 5′- P.GUCUGGU UUGCACUUGCACdTdT-3′ (antisense); *Fas*(sequence 2), beginning at nt 874, 5′-P.AGCCGAAUGUCGCAGAACCdTdT-3′ (sense); 5′-P.GGUUCUGC-GACAUU CGGCUdTdT-3′ (antisense); *Fas*(sequence 3), beginning at nt 137, 5′-P.GGAUUAUAUCAAGGAGGCCdTdT-3′ (sense); 5′-P.GGCCUCCUU GAUAUAAUCCdTdT-3′ (antisense); *Fas*(sequence 4), beginning at nt 501, 5′-P.AUCGCCUAUGGUUGUUGACdTdT-3′ (sense); 5′-PGUCAACAACCAUA GGCGAUdTdT-3′ (antisense); *Fas*(sequence 5), beginning at nt 667, 5′-P.A UACAUCCCGAGAAUUGCUdTdT-3′ (sense); 5′-P.AGCAAUUCUCG GGAU GUAUdTdT-3′ (antisense); *Fas*(sequence 6), beginning at nt 873, 5′-P.AAG CCGAAUGUCGCAGAACdTdT-3′ (sense); 5′-P.GUUCUGCGACAUUCGGC UUdTdT (antisense). *GFP*(sequence 1), 5′- P.GGCUACGUCCAGGAGCG-CACC-3′ (sense), 5′- P.UGCGCUCCUGGACGUAGCCUU-3′ (antisense); *GFP*(sequence 2), 5′-P.GGCUACGUCCAGGAGCGCAdTdT-3′ (sense), 5′-P.U GCGCUCCUGGACGUAGCCdTdT-3′ (antisense); P represents 5′ phosphate.

RNAs were deprotected and annealed according to the manufacturer's instruction. Cy5-labeled *Fas* siRNA with the fluorophore coupled to the 3′ end of the sense strand was produced by Dharmacon Research.

siRNA treatment. Male BALB/c mice, 8–10 weeks of age and weighing 20–25 g, were purchased from the Jackson Laboratory (Bar Harbor, Maine). Synthetic siRNAs were delivered *in vivo* using a modified 'hydrodynamic transfection method'[13], by which 50 μg siRNA dissolved in 1 ml PBS was rapidly injected into the tail vein. The injection was repeated 8 and 24 h later. Control mice were injected with an equal volume of normal saline or *GFP* siRNA.

Isolation of hepatocytes. Hepatocytes were isolated by a modified hepatic portal perfusion technique[29]. The purity of hepatocytes, determined by flow cytometric analysis of intracellular albumin staining using fluorescein-conjugated goat antibody against mouse albumin (Bethyl Laboratories, Montgomery, Texas), was >90% (data not shown). For some experiments, cells were briefly cultured after plating at 2×10^6 cells per 60 mm collagen-coated culture dish in William's Medium E (Gibco-BRL, Grand Island, New York) supplemented with 10% fetal bovine serum, 15 mmol/l HEPES (pH 7.4), 1 μmol/l insulin, 2 mmol/l l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

RPA. Total RNA was extracted from hepatocytes using Trizol reagent (Molecular Research Center, Cincinnati, Ohio), and an RPA was carried out using 15 μg of total RNA and the In-vitro Transcription Kit and mouse mAPO-3 multi-probe template set (BD Pharmingen, San Diego, California) according to the manufacturer's instructions. Intensities of the protected bands were quantified by phosphorimaging (Fuji-BAS 1500, Fuji, Tokyo, Japan) based on the ratios of the mRNAs of interest to *Gapdh* (internal control).

Immunoblot. Protein extracts of mouse hepatocytes were resolved over 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, probed

with rabbit polyclonal antibodies against mouse Fas and then with peroxidase-conjugated goat antibodies against rabbit as the secondary antibody (Oncogene Research Product, Boston, Massachusetts), and then visualized by chemiluminescence (Amersham Life Science, Arlington Heights, Illinois).

Apoptosis assay. Primary hepatocytes from untreated mice or mice treated with *Fas* siRNA or saline were seeded in 12-well plates at a density of 1×10^5/ml. The next day Jo2 monoclonal antibody (500 ng/ml; BD Pharmingen) was added; 24 h later, liver cell apoptosis was evaluated by FITC-labeled TUNEL assay (Boehringer Mannheim, Mannheim, Germany), analyzed by flow cytometry on a FACScan flow cytometer with LYSIS II software (Nippon Becton Dickinson, Tokyo, Japan).

ALT release assay. Target liver cells, plated in 12-well plates at 1×10^4 cells/well, were co-cultivated overnight with mononuclear cells isolated from livers of mice treated with ConA (15 mg per kg body weight) at different effector-to-target ratios. Release of ALT from hepatocytes was measured in the supernatants using an ALT assay kit (Boehringer Mannheim). Cytotoxicity was expressed as the percentage of ALT in the supernatants, compared to total ALT in detergent-lysed cells.

Induction of acute hepatitis. Mice were injected intravenously through the tail vein with ConA (15 mg/kg; Sigma, St. Louis, Missouri) reconstituted in pyrogen-free saline[23]. After 20 h, serum ALT and AST were measured using a standard clinical automatic analyzer (Hitachi, type 7150, Tokyo, Japan), and paraffin-embedded liver sections were stained with H&E. Other mice were injected intraperitoneally with 8 μg of Jo2 monoclonal antibody and followed for 10 d, then killed and their livers subjected to histological examination.

Induction of liver fibrosis. A reduced dose of ConA (8 mg/kg) dissolved in pyrogen-free saline was injected in the tail vein weekly for 6 consecutive weeks[30]. Mice were treated with three injections of siRNA as above beginning 24 h after the second and fourth Con A injections and sacrificed 1 week after the final injection of ConA. Liver fibrosis was evaluated by H&E staining and measurement of hepatic hydroxyproline[26] and serum procollagen type III using a sequential saturation radioimmunoassay (Calbiochem, La Jolla, California), as described[27].

Acknowledgments

This work was supported by US National Institutes of Health grants AI42510 and AI45406 (J.L.), AI49792 and AI45306 (P.S.), American Foundation for AIDS Research (AmfAR) grants 70589-32-RF (S.L.) and 70540-30-RF (N.I.), Guangdong Provincial Natural Science Foundation grants 980098 (E.S.) and 001359 (J.M.), and National Natural Scientific Foundation of China grants 39670705 (J.C.), 39700135 and 39970718 (J.M.).

Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 21 OCTOBER 2002; ACCEPTED 24 JANUARY 2003.

1. Hannon, G.J. RNA interference. *Nature* **418**, 244–251 (2002).
2. Rust, C. & Gores, G.J. Apoptosis and liver disease. *Am. J. Med.* **108**, 567–574 (2000).
3. Elbashir, S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
4. Caplen, N.J., Parrish, S., Imani, F., Fire, A. & Morgan, R.A. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* **98**, 9742–9747 (2001).
5. McCaffrey, A.P. *et al.* RNA interference in adult mice. *Nature* **418**, 38–39 (2002).
6. Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A. & Herweijer, H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **32**, 107–108 (2002).
7. Xia, H., Mao, Q., Paulson, H.L. & Davidson, B.L. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat. Biotechnol.* **20**, 1006–1010 (2002).
8. Kondo, T., Suda, T., Fukuyama, H., Adachi, M. & Nagata, S. Essential roles of the Fas ligand in the development of hepatitis. *Nat. Med.* **3**, 409–413 (1997).
9. Siegel, R.M. & Fleisher, T.A. The role of Fas and related death receptors in autoimmune and other disease states. *J. Allergy Clin. Immunol.* **103**, 729–738 (1999).
10. Ogasawara, J. *et al.* Lethal effect of the anti-Fas antibody in mice. *Nature* **364**, 806–809 (1993).
11. Li, X.K. *et al.* Fulminant hepatitis by Fas-ligand expression in MRL-lpr/lpr mice grafted with Fas-positive livers and wild-type mice with Fas-mutant livers. *Transplantation* **71**, 503–508 (2001).
12. Canbay, A. *et al.* Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis. *Gastroenterology* **123**, 1323–1330 (2002).
13. Zhang, G., Budker, V. & Wolff, J.A. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10**, 1735–1737 (1999).
14. Zhang, H. *et al.* Reduction of liver Fas expression by an antisense oligonucleotide protects mice from fulminant hepatitis. *Nat. Biotechnol.* **18**, 862–867 (2000).
15. Bertrand, J. *et al.* Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. *Biochem. Biophys. Res. Commun.* **296**, 1000– 1004 (2002).
16. Schwarz, D.S., Hutvagner, G., Haley, B. & Zamore, P.D. Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Mol. Cell* **10**, 537–548 (2002).
17. Ketting, R.F. *et al.* Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654–2659 (2001).
18. Lipardi, C., Wei, Q. & Paterson, B.M. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* **107**, 297–307 (2001).
19. Takahashi, T. *et al.* Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* **76**, 969–976 (1994).
20. Ryo, K. *et al.* Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. *Am. J. Gastroenterol.* **95**, 2047–2055. (2000).
21. Faouzi, S. *et al.* Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-κB-independent, caspase-3-dependent pathway. *J. Biol. Chem.* **276**, 49077–49082 (2001).
22. Seino, K. *et al.* Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology* **113**, 1315–1322 (1997).
23. Takeda, K. *et al.* Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc. Natl. Acad. Sci. USA* **97**, 5498–5503 (2000).
24. Miyazawa, Y., Tsutsui, H., Mizuhara, H., Fujiwara, H. & Kaneda, K. Involvement of intrasinusoidal hemostasis in the development of concanavalin A-induced hepatic injury in mice. *Hepatology* **27**, 497–506 (1998).
25. Galle, P.R. *et al.* Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J. Exp. Med.* **182**, 1223–1230 (1995).
26. Takahashi, S. & Lee, M.J. Collagen metabolism in fibrotic liver. Effects of concanavalin A and aggregated myeloma immunoglobin G. *Biochem. J.* **241**, 49–54 (1987).
27. Badawy, A.A. *et al.* Evaluation of colchicine with or without praziquantel therapy in the control of hepatic fibrosis in murine schistosomiasis. *Pharmacol. Res.* **33**, 319–325 (1996).
28. Dhiman, R.K., Seth, A.K., Jain, S., Chawla, Y.K. & Dilawari, J.B. Prognostic evaluation of early indicators in fulminant hepatic failure by multivariate analysis. *Dig. Dis. Sci.* **43**, 1311–1316 (1998).
29. Klauning, J.E. *et al.* Mouse liver cell culture. 1. Hepatocyte isolation. *In Vitro* **17**, 913–925 (1981).
30. Kimura, K. *et al.* Immunopathogenesis of hepatic fibrosis in chronic liver injury induced by repeatedly administered concanavalin A. *Int. Immunol.* **11**, 1491–1500 (1999).

© 2003 Nature Publishing Group http://www.nature.com/naturemedicine